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Collection of *Lupinus angustifolius* L. germplasm and characterisation of morphological and molecular diversity

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Abstract

Lupinus angustifolius L. is a Mediterranean species, domesticated in the 20th century, representing an important grain legume crop in Australia and other countries. This work is focused on the collection of wild germplasm and on the characterisation of morphological and molecular diversity of germplasm accessions. It reports the collection of 81 wild *L. angustifolius* accessions from the South and Centre of Portugal, available at the 'Instituto Superior de Agronomia Gene Bank', with subsequent morphological and molecular characterisation of a selection of these and other accessions. A multivariate analysis of morphological traits on 88 *L. angustifolius* accessions (including 59 wild Portuguese accessions, 15 cultivars and 14 breeding lines) showed a cline of variation on wild germplasm, with plants from Southern Portugal characterised by earlier flowering, higher vegetative development and larger seeds. AFLP and ISSR molecular markers grouped modern cultivars as sub-clusters within the wider diversity of wild germplasm, revealing the narrow pool of genetic diversity on which domesticated accessions are based. The importance of preserving, characterising and using wild genetic resources for *L. angustifolius* crop improvement is outlined by the results obtained.

Introduction

The species *Lupinus angustifolius* L. (narrow leaf lupin), originated from the Mediterranean basin, is an important grain legume crop in different parts of the world. Over one million hectares are nowadays cultivated in Western Australia (Russell 2000), yielding about 1 t ha^{-1}, this value increasing at an average rate of 1.3% per year (Cowling and Stephens 1997). Other important cultivation areas are located in Poland (ca. 60,000 ha), South Africa (ca. 30,000 ha) and Chile (5000–11,000 ha), being the total world grain production of sweet narrow leaf lupins estimated in about 1.8 million tonnes per year, representing 3% of total world pulse production (Cowling et al. 1998b). As compared to other pulses, lupin seeds have a high protein content (ca. 32%) and low levels of most anti-nutritional factors (common in other legumes), except for alkaloids, which are removable genetically (sweet cultivars) or by processing (Petterson 1998).

Although previously cultivated as a forage crop, *L. angustifolius* was only recently fully domesticated, based on non-shattering plants with sweet permeable seeds obtained by mutation in Germany

in the early decades of the 20th century by Reinhold von Sengbusch (Cowling et al. 1998a). A strong effort has been made to enlarge the genetic diversity on which current cultivars are based. This has been done by means of crossing breeding lines, arising from that mutation programme, with diverse wild germplasm. Hence, the collection and study of the diversity of wild *L. angustifolius* in its natural distribution area is of great importance to the expansion of the crop. *Lupinus angustifolius* is not a current crop in the areas were it is naturally distributed (circum-Mediterranean area), unlike *L. luteus* L. (yellow lupin) and *L. albus* L. (white lupin). Therefore, genetic diversity in *L. angustifolius* seems to be larger than that found both in *L. luteus* and *L. albus* (Talhinhas 2002). This would be easily explained by a lack of selection pressure in *L. angustifolius* towards neighbouring crops of domesticated or semi-domesticated forms, as it happened with *L. luteus* and *L. albus* crops in the Mediterranean area in relation to their wild relatives.

Lupinus angustifolius is more frequently found in the Iberian Peninsula, Morocco and islands and coasts of Aegean Sea, although the species is putatively originated from the coastal areas of Turkey and Syria (Gladstones 1998). *Lupinus angustifolius* can be found from sea level to about 2000 m high, in areas of annual average rainfall ranging 200–1500 mm and soil pH ranging 4.2–9.0 (Buirchell and Cowling 1998). Partial or total resistance to different diseases are reported from wild accessions from various origins. Plant morphology is known to vary around the Mediterranean basin, with Western accessions generally more late flowering, taller, with smaller seeds and leaves, lesser seed production, seed oil content and water stress tolerance, as compared to East Mediterranean accessions (Kurlovich 1994; Święcicki and Święcicki 1995).

The use of molecular markers was shown to be useful for characterising genetic diversity in *L. albus* germplasm collections (Qiu et al. 1995; Gilbert et al. 1999), which can be helpful for pointing out possible redundant accessions and, on the other hand, for picking up contrasting genotypes.

The aim of this work was to enlarge the 'Instituto Superior de Agronomia Gene Bank' collection with wild *L. angustifolius* accessions from the South and Centre of Portugal. The genetic diversity of the *L. angustifolius* collection was then studied, using subsets of the collection covering wild and domesticated accessions, respectively from different geographic origins and from different breeding programmes. Morphological characterisation was conducted on a field trial under a Mediterranean climate. Molecular diversity was accessed using AFLP and ISSR molecular markers.

Materials and methods

Germplasm collection and plant material

Seeds from wild *L. angustifolius* plants were collected in May–June 1997 in the South and Centre of Portugal, after a preliminary survey at flowering time (February–March) for easier location of plants. A total of 81 accessions were obtained either as populations or as single plant descendents, and several collection data were recorded. Seed stocks are kept at the Instituto Superior de Agronomia (ISA) germplasm bank.

Other plant material used for characterisation includes 52 accessions from different sources (as reported in Table 1), including wild germplasm previously collected, breeding lines and the following cultivars: Belara; Danja; Frost; Gungurru; Illyarrie; Jak; Kalya; Marri; Merrit; Moredou; Myallie; Rancher; Rubine; Steb; Ster; Tallerack; Unicrop; Uniharvest; Warrah; Wonga; Yandee; Yorrel.

Morphological characterisation

Experimental design

For the morphological characterisation, plants were grown on a field trial. Eighty-eight *L. angustifolius* accessions (61 representing wild germplasm and 27 representing cultivars and breeding lines; listed in Figure 3) were cultivated in randomised rows (50 seeds per row 10 cm apart, rows 40 cm apart from each other). Each 10th row was sown with a control line (*L. angustifolius* 'Illyarrie'). Trial was carried out in Lisbon, Portugal (38°42′ N, 9°11′ W, 60 m), on a vertisoil (neutral pH).

Data collection

Several characterisation data and some evaluation data were obtained (Table 2), according to the *Lupinus* descriptor (IBPGR 1981). Ten values were obtained per accession for most quantitative

Table 1. Accessions under analysis, including main passport and collection data for wild accessions [a]

1.1 Accession number [b]	1.6 Name assigned by collector	2.1 Institution (and collectors) [c]	2.3 Collection year	2.5b Collection site	2.6 Latitude (N)	2.7 Longitude (W)	2.9 Altitude (m)	2.10 Sample origin [d]	2.11 Sample type [e]	2.13a General topography [f]	2.13b Local topography [g]
Wild accessions											
10095	Alt97-67	ISA (PT)	1997	Juromenha, Alandroal	38°45′	7°13′	160	2	1	4	2
10096	Alt97-68	ISA (PT)	1997	Juromenha, Alandroal	38°45′	7°13′	160	2	1	4	2
10097	Alt97-69	ISA (PT)	1997	Juromenha, Alandroal	38°45′	7°13′	160	2	1	4	2
10135	EAN 1048	EAN (EP,LG)	1980	Alter do Chão	39°13′	7°38′	400	2	1	5	3
10051	Alt97-27	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10052	Alt97-28	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10055	Alt97-30	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10056	Alt97-31	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10057	Alt97-32	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10058	Alt97-33	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10059	Alt97-34	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10060	Alt97-35	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10061	Alt97-36	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10062	Alt97-37	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10063	Alt97-38	ISA (PT)	1997	Arronches	39°08′	7°17′	280	2	1	3	8
10064	Alt97-39	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10066	Alt97-40	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10067	Alt97-41	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10068	Alt97-42	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10069	Alt97-43	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10070	Alt97-44	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10071	Alt97-45	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10072	Alt97-46	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°22′	320	2	1	3	8
10073	Alt97-47	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°21′	340	2	1	4	6
10074	Alt97-48	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°21′	340	2	1	4	6
10075	Alt97-49	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7 °21′	340	2	1	4	6
10077	Alt97-50	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°21′	340	2	1	4	6
10078	Alt97-51	ISA (PT)	1997	Assumar (prox.), Arronches	39°07′	7°21′	340	2	1	4	6
10143	EAN 948	EAN (C,EB)	1980	Degolados (prox.), Arronches	39°05′	7°10′	300	2	1	–	–
10149	EAN 984	EAN (C,EB)	1980	Caria, Belmonte	40°17′	7°21′	450	2	1	5	3
10150	EAN 986	EAN (C,EB)	1980	Caria, Belmonte	40°20′	7°21′	600	2	1	5	3
10116	BI97-12	EAN/ISA (JNM,LG,PT)	1997	Castelo Branco	39°52′	7°25′	370	2	1	3	1
10118	BI97-2	EAN/ISA (JNM,LG,PT)	1997	Castelo Branco	39°52′	7°25′	400	2	1	3	1
10127	BI97-4	EAN/ISA (JNM,LG,PT)	1997	Castelo Branco	39°52′	7°25′	380	2	1	3	1
10128	BI97-5	EAN/ISA (JNM,LG,PT)	1997	Castelo Branco	39°52′	7°25′	380	2	1	3	1
10114	Benavente	ISA (JNM)	–	Benavente	–	–	–	2	1	–	–
10129	BI97-9	EAN/ISA (JNM,LG,PT)	1997	Castelo Branco	39°52′	7°25′	370	2	1	3	1
10139	EAN 1067	EAN (EP,LG)	1980	Escalos, Castelo Branco	39°52′	7°26′	350	2	1	4	3

Table 1. Continued

1.1 Accession number [b]	1.6 Name assigned by collector	2.1 Institution (and collectors) [c]	2.3 Collection year	2.5b Collection site	2.6 Latitude (N)	2.7 Longitude (W)	2.9 Altitude (m)	2.10 Sample origin [d]	2.11 Sample type [e]	2.13a General topography [f]	2.13b Local topography [g]
10120	BI97-23	EAN/ISA (JNM,LG,PT)	1997	Orjais, Covilhã	40°20′	7°23′	550	2	1	7	6
10044	Alt97-20	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10045	Alt97-21	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10046	Alt97-22	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10047	Alt97-23	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10048	Alt97-24	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10049	Alt97-25	ISA (PT)	1997	Crato	39°15′	7°37′	200	2	1	4	8
10136	EAN 1050	EAN (EP,LG)	1980	Crato	39°17′	7°35′	200	2	1	5	3
10090	Alt97-62	ISA (PT)	1997	Juromenha (prox.), Elvas	38°46′	7°13′	200	2	1	4	8
10091	Alt97-63	ISA (PT)	1997	Juromenha (prox.), Elvas	38°46′	7°13′	200	2	1	4	8
10092	Alt97-64	ISA (PT)	1997	Juromenha (prox.), Elvas	38°46′	7°13′	200	2	1	4	8
10093	Alt97-65	ISA (PT)	1997	Juromenha (prox.), Elvas	38°46′	7°13′	200	2	1	4	8
10094	Alt97-66	ISA (PT)	1997	Juromenha (prox.), Elvas	38°46′	7°13′	200	2	1	4	8
10050	Alt97-26	ISA (PT)	1997	Sta. Eulália, Elvas	39°01′	7°14′	220	2	1	3	1
10079	Alt97-52	ISA (PT)	1997	Sta. Eulália, Elvas	39°02′	7°16′	250	2	1	3	8
10080	Alt97-53	ISA (PT)	1997	Sta. Eulália, Elvas	39°02′	7°16′	250	2	1	3	8
10081	Alt97-54	ISA (PT)	1997	Sta. Eulália, Elvas	39°02′	7°16′	250	2	1	3	8
10082	Alt97-55	ISA (PT)	1997	Sta. Eulália, Elvas	39°02′	7°16′	250	2	1	3	8
10083	Alt97-56	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10084	Alt97-57	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10085	Alt97-58	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10086	Alt97-59	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10088	Alt97-60	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10089	Alt97-61	ISA (PT)	1997	Sta. Eulália, Elvas	39°00′	7°15′	270	2	1	3	8
10098	Alt97-70	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10099	Alt97-71	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10100	Alt97-72	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10101	Alt97-73	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10102	Alt97-74	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10103	Alt97-75	ISA (PT)	1997	Courelas da Toura, Évora	38°39′	7°42′	250	2	1	3	8
10122	BI97-27	EAN/ISA (JNM,LG,PT)	1997	Castelo Rodrigo, Fig. Cast. Rodrigo	40°52′	6°55′	700	2	2	6	8
10123	BI97-28	EAN/ISA (JNM,LG,PT)	1997	Castelo Rodrigo, Fig. Cast.Rodrigo	40°52′	6°55′	700	2	2	6	8
10148	EAN 982	EAN (C,EB)	1980	Fundão	40°09′	7°28′	470	2	1	4	1
10142	EAN 896	EAN (EB,JNM,MB)	1980	Ameixial, Loulé	37°21′	7°58′	350	2	1	5	6
10147	EAN 965	EAN (C,EB)	1980	Alvarrões, Marvão	39°22′	7°24′	650	2	1	5	3
10035	Alt97-1	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1
10043	Alt97-2	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1
10054	Alt97-3	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1
10065	Alt97-4	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1
10076	Alt97-5	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1

10087	Alt97-6	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	4	1
10053	Alt97-29	ISA (PT)	1997	S. Salvador, Marvão	39°22′	7°23′	600	2	1	3	8
10140	EAN 859	EAN (EB,EP,MB)	1980	Ponte Samouco, Monchique	37°16′	8°36′	200	2	1	8	5
10141	EAN 860	EAN (EB,EP,MB)	1980	João de Gales, Monchique	37°16′	8°35′	200	2	1	5	5
10138	EAN 1057	EAN (EP,LG)	1980	Alpalhão, Nisa	39°25′	7°36′	320	2	1	4	1
10125	BI97-30	EAN/ISA (JNM,LG,PT)	1997	Freixeda, Pinhel	40°42′	7°09′	750	2	1	4	8
10126	BI97-33	EAN/ISA (JNM,LG,PT)	1997	Freixeda, Pinhel	40°42′	7°09′	750	2	1	4	8
10121	BI97-25	EAN/ISA (JNM,LG,PT)	1997	Pinhel	40°49′	7°03′	470	2	1	6	2
10124	BI97-29	EAN/ISA (JNM,LG,PT)	1997	Ribeira da Pega, Pinhel	40°45′	7°05′	580	2	1	5	9
10144	EAN 952	EAN (C,EB)	1980	Alegrete, Portalegre	39°14′	7°19′	450	2	1	5	3
10145	EAN 954	EAN (C,EB)	1980	Hortas, Portalegre	39°16′	7°22′	450	2	1	5	3
10146	EAN 958	EAN (C,EB)	1980	Hortas, Portalegre	39°17′	7°23′	500	2	1	5	1
10036	Alt97-13	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10037	Alt97-14	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10038	Alt97-15	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10039	Alt97-16	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10040	Alt97-17	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10041	Alt97-18	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10042	Alt97-19	ISA (PT)	1997	Monte Paleiros, Portalegre	38°20′	7°24′	500	2	1	8	3
10137	EAN 1054	EAN (EP,LG)	1980	Portalegre	39°17′	7°26′	350	2	1	8	6
10181	Ranginha	ISA (JNM)	–	Ranginha, Vila Real	–	–	–	2	1	–	–
Breeding lines											
10004	28439	AWA (WC)	–	Australia	–	–	–	–	5	–	–
10005	28829	AWA (WC)	–	Australia	–	–	–	–	5	–	–
10013	84A:509	AWA (WC)	–	Australia	–	–	–	–	5	–	–
10200	WTD-W-17	–	–	Germany	–	–	–	–	5	–	–
10014	A80	–	–	Italy	–	–	–	–	5	–	–
10132	EAN	–	–	Portugal	–	–	–	–	5	–	–
10001	3075	ENMP (MTS)	–	Spain	–	–	–	–	5	–	–
10002	3082	ENMP (MTS)	–	Spain	–	–	–	–	5	–	–
10003	3084	ENMP (MTS)	–	Spain	–	–	–	–	5	–	–
10160	Jambrina 1	–	–	Spain	–	–	–	–	5	–	–
10161	Jambrina 2	–	–	Spain	–	–	–	–	5	–	–
10162	Jambrina 3	–	–	Spain	–	–	–	–	5	–	–
Cultivars											
10112	Belara	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10151	Frost	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10163	Kalya	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10166	Marri	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10168	Merrit	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10171	Myallie	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10180	Rancher	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10195	Tallerack	AWA (WC)	–	Australia	–	–	–	–	6	–	–

Table 1. Continued

1.1 Accession number [b]	1.6 Name assigned by collector	2.1 Institution (and collectors) [c]	2.3 Collection year	2.5b Collection site	2.6 Latitude (N)	2.7 Longitude (W)	2.9 Altitude (m)	2.10 Sample origin [d]	2.11 Sample type [e]	2.13a General topography [f]	2.13b Local topography [g]
10197	Uniharvest	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10198	Warrah	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10199	Wonga	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10201	Yandee	AWA (WC)	–	Australia	–	–	–	–	6	–	–
10131	Danja	–	–	Australia	–	–	–	–	6	–	–
10157	Gungurru	–	–	Australia	–	–	–	–	6	–	–
10158	Illyarrie	–	–	Australia	–	–	–	–	6	–	–
10169	Moredou	–	–	Australia	–	–	–	–	6	–	–
10196	Unicrop	–	–	Australia	–	–	–	–	6	–	–
10202	Yorrel	–	–	Australia	–	–	–	–	6	–	–
10184	Rubine	–	–	Germany	–	–	–	–	6	–	–
10159	Jak	–	–	South Africa	–	–	–	–	6	–	–
10191	Steb	–	–	South Africa	–	–	–	–	6	–	–
10192	Ster	–	–	South Africa	–	–	–	–	6	–	–

[a] Characters with numbers refer to the species descriptor (IBPGR 1981); accessions are sorted by type and by alphabetic order of origin.
[b] Accessions in bold were collected within the mission reported here.
[c] 2.1 Institutions: AWA – Agriculture West Australia, Australia; EAN – Estação Agronómica Nacional, Portugal; ISA – Instituto Superior de Agronomia, Portugal; UAç – Universidade dos Açores, Portugal. Collectors: C – Casanova; DM – Duarte Marques; EB – Eliseu Bettencourt; EP – Eugene Pascual; JNM – João Neves-Martins; JR – Joaquim Rocha; LG – Luis Gusmão; MB – M. Bueno; MTS – Manuel Tavares de Sousa; PT – Pedro Talhinhas; SC – Santos Costa; WC – Wallace Cowling.
[d] 2.10 Sample origin: 1 – undisturbed area; 2 – disturbed uncultivated area; 3 – cultivated area; 4 – farm warehouse; 5 – market; 6 – agricultural institution; 7 – other.
[e] 2.11 Sample type: 1 – wild; 2 – weed; 3 – semi-cultivated; 4 – landrace; 5 – breeding line; 6 – modern cultivar.
[f] 2.13a General topography: 1 – swamp; 2 – flood plain; 3 – plain level; 4 – undulating; 5 – hilly; 6 – high hills; 7 – very steppy; 8 – high mountain.
[g] 2.13b Local topography: 1 – level; 2 – summit; 3 – cliff; 4 – hill top; 5 – top of slope; 6 – mid-slope; 7 – terrace; 8 – bottom of slope; 9 – wide valley; 10 – narrow valley.

Table 2. Characters studied for morphological analysis (sorted according to measuring time) and limits of variation observed

Descriptor reference [a]	Character [b]	Acronym	Min.	Mean	Max.	Unit
Quantitative characters						
4.6.26	Flower length	FL	10.6	11.8	13.2	mm
6.2	No. of days to flowering	NDF	92	111	139	no.
–	No. of leaves on main stem	NL	15	30	40	no.
–	Length of central leaflet [c]	LLl	17.9	28.9	38.2	mm
–	Width of central leaflet [c]	WLl	2.1	3.6	5.1	mm
4.5.7	No. of leaflets per leaf [c]	NLl	6	7	9	no.
4.5.10	Stipule length [c]	SpL	4.820	6.750	8.733	mm
–	Stipule width [c]	SpW	0.6	1.0	1.4	mm
4.5.13	Petiole length [c]	PL	6.3	31.6	44.5	mm
–	Petiole width [c]	PW	0.8	1.2	1.5	mm
4.3.6	Stem diameter [c]	SD	2.7	3.9	6.3	mm
4.7.2	Pod length [d]	PdL	30.0	39.1	50.5	mm
4.7.3	Pod width [d]	PdW	6.1	9.6	13.3	mm
6.6	Height from soil to 1st pod	H1Pd	11.9	22.8	33.0	cm
4.4.2	No. of primary branches	N1B	3	7	15	no.
–	Length of primary branch no. i	L1B–i	–	–	–	cm
–	Basal branches	BB	0	3	5	no.
–	No. podsets on branches order i	NPsOi	–	–	–	no.
–	Total no. of pods per podset	TNPd/TNPs	1.000	2.135	3.869	no.
–	Total no. of seeds per pod	TNSd/TNPd	0.05974	1.305	3.003	no.
–	Total thousand seeds weight	TThSdWe	45.7	107.5	197.5	g
–	Seed length	SdL	4.5	6.1	7.3	mm
–	Seed width	SdW	3.9	5.2	6.7	mm
–	Seed thickness	SdTh	3.1	4.2	5.5	mm
Qualitative characters						
4.6.5a	Keel tip colour before open flower	KTCBO1	–	–	–	–
4.6.5b	2nd keel tip colour before open flower	KTCBO2	–	–	–	–
4.6.5c	Rest of keel colour before open flower	RKCBO	–	–	–	–
4.6.15	Keel colour before wilting	KCBWt	–	–	–	–
4.6.13	Standard colour before wilting	SdCBWt	–	–	–	–
4.7.6	Pod shattering	PdSh	–	–	–	–

[a] (IBPGR 1981).
[b] Other characters were recorded but removed from effective statistical analyses due to environmental variability (inflorescence length, leaf diameter, total plant height, no. of pods, no. of seeds, seeds weight).
[c] Measurements made on the upper leaf of the main stem.
[d] Measurements made on the first (lower) pod of the main stem.

characters (30 for seed dimensions) while a single value per accession was recorded for qualitative characters, as recommended previously (IBPGR 1981; Martins 1991).

Some traits measured as qualitative where transformed into quantitative and included in the analyses. They were: the colour of the keel just before opening (the tip, the strip before the tip and the remaining keel); the colour of the keel just before wilting; the standard colour of just open flower and just before wilting; pod shattering. Flower organs colours where quantified on the basis of the intensity of anthocyanin pigmentation (0-white; 1-pink; 2-blue/violet). Pod shattering was quantified as in the lupin descriptor (IBPGR 1981): 0-non-shattering; 3-slight shattering; 5-moderate shattering; 7-severe shattering.

Data analysis

For each character, the accessions were compared using Analysis of Variance (ANOVA) of data, followed by average comparison tests (Tukey Honest Significant Difference), both at 95% confidence (Statistica 5.0, StatSoft Inc.). Average values for each pair 'accession' × 'character' were used for multivariate analysis, aiming to establish patterns of relationship among the accessions.

An ANOVA was also performed for the different characters recorded on the control line. Another measure of environmental invariability was

obtained by calculating the *sensu lato* heritability ($h^2 = (s_P^2 - s_A^2)/s_P^2$). For each character, the phenotypic variance ($s^2{}_P$) is calculated on the average values for each accession, while environmental variance ($s^2{}_A$) is the average of the variances calculated from each accession replicates. These procedures were carried out in order to find and eliminate those characters showing environmental variability throughout the trial as well as those with low heritability.

Multivariate analysis (using NTSYSpc 2.01b, Applied Biostatistics Inc.) was performed to calculate the average Euclidian distances between each pair of accessions, then compiled into a matrix. Data in this matrix was then used both for Cluster Analysis (CA) and Principal Component Analysis (PCA).

In CA, the accessions were clustered into hierarchical groups represented in a dendrogram according to the Unweighted Pair Group Method using Arithmetic Averages (UPGMA), again using NTSYSpc 2.01b. A cophenetic correlation coefficient was calculated to represent the degree of information lost when converting the original distance matrix into a dendrogram.

In PCA, a multidimensional data set (n characters $\times$ p accessions) was reduced to a 2- or 3-dimensional representation, projecting the original standardised data into an axis system obtained by calculated Eigen vectors and values from distance matrix (Dunn and Everitt 1982), using NTSYSpc 2.01b.

Correlation coefficients were calculated between characters, compiled into matrix and submitted to CA and PCA.

Molecular characterisation

Representing the groups obtained from morphological studies, 35 accessions were chosen for AFLP and ISSR analyses, among which 25 were wild germplasm and 10 were cultivars and breeding lines (listed in Figure 3).

DNA extraction

For each accession, DNA was extracted from two individual plants. Four to five leaves of each plant were ground in mortar in the presence of liquid nitrogen and DNA extracted as previously (Talhinhas et al. 2003) and resuspended in TE.

AFLP

The entire process of digestion, adaptors ligation, preamplification and amplification was done using the "AFLP Starter Primer Kit" (Invitrogen) (Vos et al. 1995). Each reaction was repeated at least once. Two primer combinations were used for AFLP analysis (*E*-ACG/*M*-CAT and *E*-AAC/*M*-CTG).

ISSR

Three ISSR (Zietkiewicz et al. 1994) primers ordered from Invitrogen were used ($(AC)_8$YA, $(AC)_8$YG and $(CA)_8$RG) where R = A + G and Y = C + T) as previously reported (Talhinhas et al. 2003).

Data analysis

Presence/absence of bands for each accession was scored visually. Dice coefficient was used to calculate a matrix for the distances between accessions (Nei and Li 1979), using NTSYSpc 2.01b. A dendrogram was obtained by UPGMA after cluster analysis (NTSYSpc 2.01b). A bootstrap analysis (using Winboot; Immanuel Yap, IRRI; http://www.irri.org/winboot.html) was performed on 100 data replicates.

Results

Germplasm collection data

Most wild *L. angustifolius* accessions were collected on road edges. Notably, *L. angustifolius* plants were far more frequent on the road edge than on the surrounding fields, either being annual or perennial agricultural fields, pastures, forests or undisturbed lands. Only accessions 10122 and 10123 were collected as weeds, on a wheat crop at Figueira de Castelo Rodrigo (altitude 700 m). The lowest collection altitude was 160 m at Jorumenha, Alandroal, while the highest was 750 m at Freixeda, Pinhel. Most accessions (67%) were collected at altitudes ranging 200–400 m, but 13% were obtained at over 600 m. About half the accessions were collected at plain level sites, but some 16% were obtained at hilly to mountainous areas, the remaining coming from sites with undulated topography. Latitude of collection sites ranges from 38°39′ N (Courela da Toura, Évora) to 40°52′ N (Castelo Rodrigo, Figueira de Castelo Rodrigo). Accessions obtained are listed in Table 1

as well as most relevant collection data. Figure 1 shows geographic location of collection sites.

Morphological diversity

Selection of characters

The heritability for each character together with ANOVA and averages comparison tests on control lines allowed the selection of a set of characters to be analysed, presenting high heritability and low environmental variation throughout the trial. Characters concerning higher branching order were not included in the analysis because of their low heritability and high environmental variation. A total of 62 characters were subject of univariate analysis. From each pair of characters

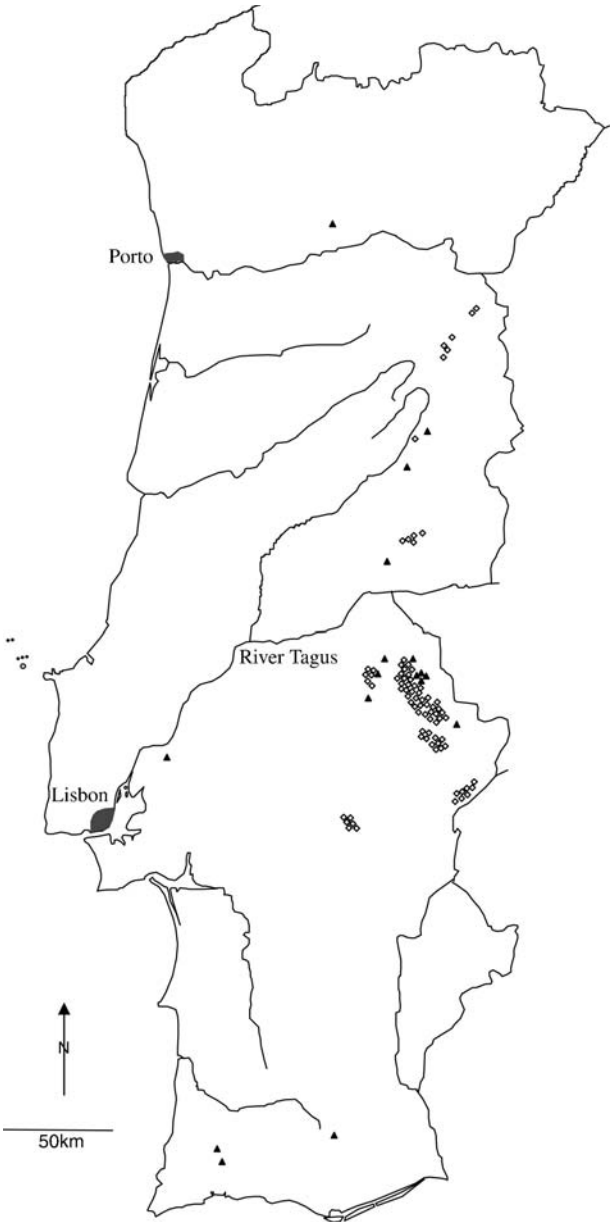

Figure 1. Map of Portugal representing the collection sites of *Lupinus angustifolius* accessions (▲ – previously collected; ◇ – collected within the present work); Lisbon – latitude 38°42′ N, latitude 9°11′ W.

presenting correlation coefficients (r_{ij}) higher than 0.95, only one character was chosen for multivariate analyses. A total of 31 characters were subject of multivariate analysis.

Using the matrix of correlations between characters, a dendrogram was calculated by CA, representing the degree of those relationships (not shown). By PCA, a 3-dimensional projection of Eigen vectors and values was obtained (Figure 2).

Bringing together both CA and PCA analyses of characters, three main groups can be defined. Group A gathers several characters related to vegetative development of plants (Figure 2). Within group A, sub-group A1 hosts traits related to plant size (length of main stem, number and length of branches, number of main stem leaves and total number of pod sets). Sub-group A2 gathers different characters related to leaf morphology and development (leaflet, petiole and stipule dimensions). Group B contains characters related to the levels of anthocyanin pigmentation on the different flower organs but also pod shattering, both being typical traits of wild germplasm. Number of days to flowering is less correlated to group B. Group C clusters characters concerning reproductive development, namely seed and pod dimensions.

These four axes of variability (A1, A2, B and C) have relatively high levels of independence among them. They represent the poles of diversity on which the different studied accessions are based.

Analysis of accessions

According to CA of the 31 characters, a dendrogram was produced for the 88 accessions studied (Figure 3). Two bi-dimensional projections were produced after PCA (not shown).

Simultaneous analysis of CA and PCA results shows that accessions can be clustered into the following groups according to character analysis:

Group 1. Low to average number of main stem leaves, large leaves; tall to very tall plants (high values for main stem height as well as number and length of primary branches); high values for reproductive development indexes (thousand seeds weight, seed dimensions, number of seeds per pod and pod dimensions), low levels of anthocyanin pigmentation in flower organs. This group includes several cultivars (namely forage ones) and breeding lines (namely European ones) exhibiting several domestication traits, such as large seeds with permeable seed coat, white flowers, non-shattering pods, but plants are tall with abundant branching.

Group 2. Includes a large number of accessions. These have only a few traits in common, such as high levels of anthocyanin pigmentation on flower organs, severe pod shattering and the impermeability of seed coat. It clusters all wild accessions and can be further subdivided into:

- sub-group 2a – tall main stem and high number of main stem leaves but low levels for reproductive development indexes (small seeds and pods); it includes 26 accessions

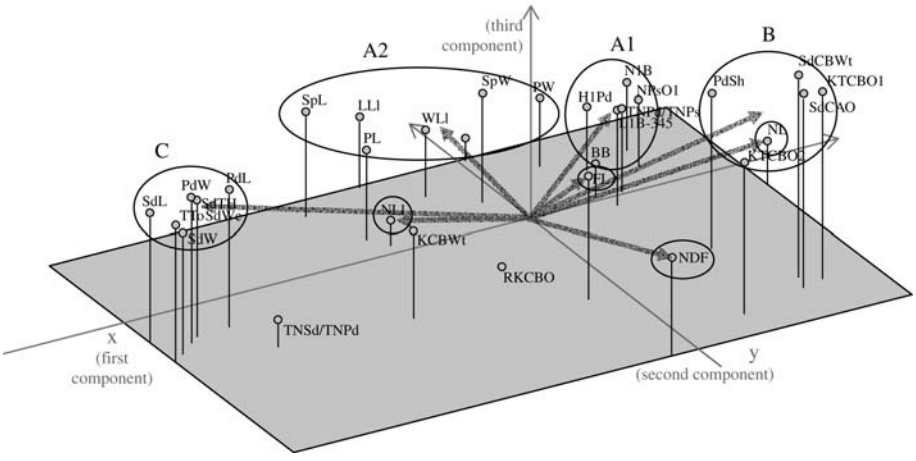

Figure 2. Projection in a 3-dimensional space of Eigen vectors and values related to characters used in multivariate analyses, obtained by principal component analysis on correlation matrix; main groups and respective vectors are shown; legend for acronyms in Table 2.

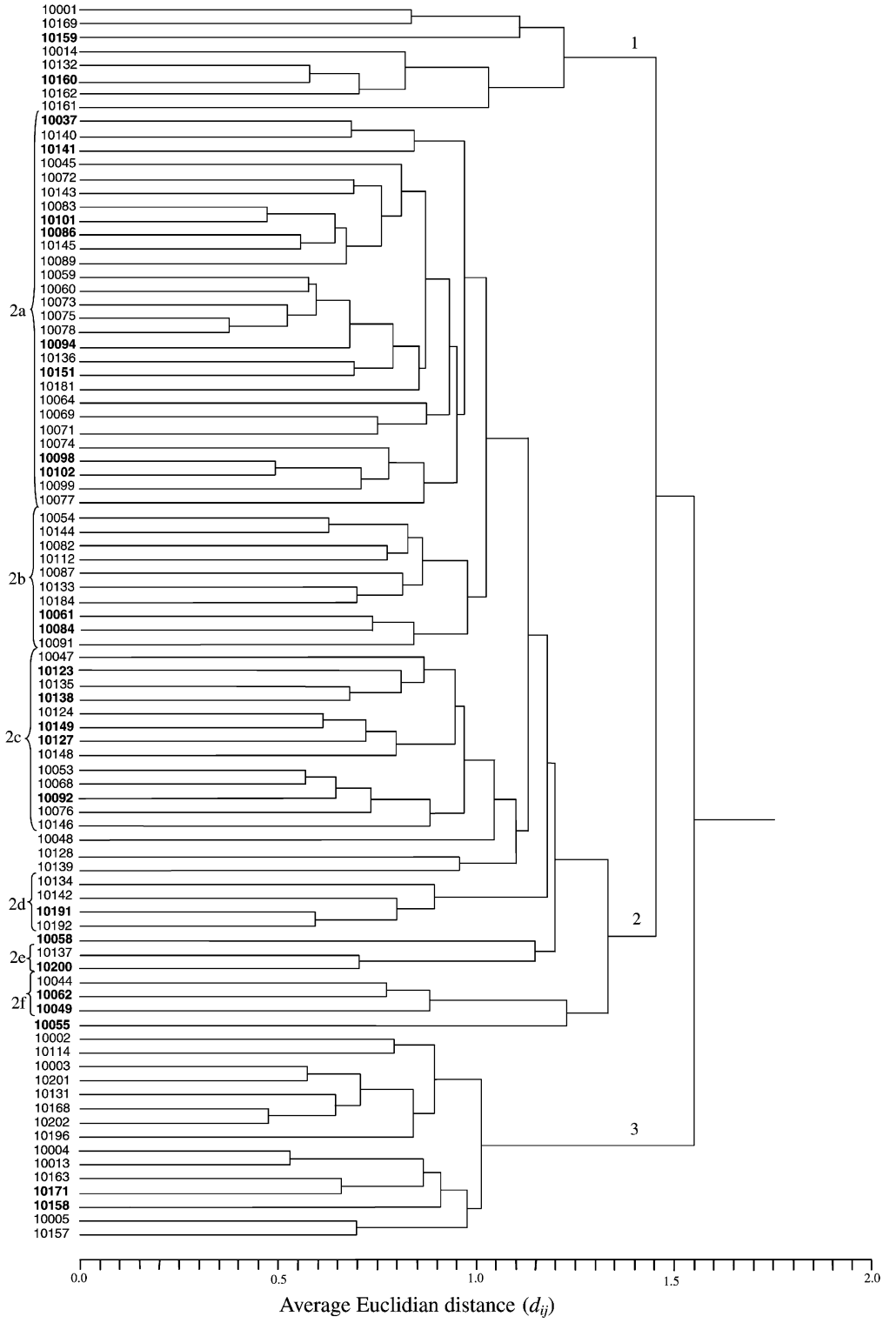

Figure 3. Dendrogram relating the 88 *Lupinus angustifolius* accessions used for multivariate analysis of morphological traits; calculated upon clustering of accessions from a distance matrix; cophenetic correlation coefficient $r = 0.74776$; accessions in bold were selected for molecular analyses.

from the South and one from the North and also the cultivar 'Frost';
- sub-group 2b – average to high values for reproductive development indexes, namely seed and pod dimensions;
- sub-group 2c – average to low values for both vegetative and reproductive development indexes and very late flowering; this group clusters several accessions from the Centre and North;
- sub-group 2d – average to low height (average main stem height and low number of primary branches) but average to large seeds and pods; it contains two cultivars and two wild accessions;
- sub-group 2e – very late flowering plants, average to large seeds, intermediate plant height; includes a German breeding line and two wild accessions from the South;
- sub-group 2f – similar to sub-group 2a, but presents extreme values for several characters, such as a very high number of main stem leaves, main stem height and primary branches number and length; late flowering plants, small seeds and pods; it includes four wild accessions;

Group 3. Low values for height and number of leaves on main stem, low number and length of primary branches, very large seeds and pods; flower organs are white and pods are non-shattering; it includes nearly all cultivars and some breeding lines.

Individual analysis of each character (univariate analysis), also leads to the three previously described main groups of accessions. Forage cultivars are taller and later flowering, but in general also have large pods and seeds. Wild accessions present a larger range of variability, but are generally taller, later flowering and have smaller pods and seeds as compared to cultivars. Within wild accessions, several characters are useful for describing a geographic cline of variation. Accessions from the South tend to have higher values for vegetative development indexes (namely primary branch length), to be earlier flowering and to have larger pods and seeds than those from the Centre. Seed size is the clearest separation among wild accessions. Those from Centre and North typically have 'thousand seeds weight' values below 68 g, while accessions from the South present values over 68 g. Most modern cultivars are characterised by presenting short and early flowering plants with large pods and seeds. The range of variation obtained for the different characters under analysis is presented in Table 1.

Some accessions are particularly meaningful for their traits:
- Spanish breeding line 10001 (group 1) showed high levels of seed production;
- accession 10122 (from Castelo Rodrigo; group 2b) presents simultaneously high levels of vegetative development, large seeds and high levels of seed production;
- accession 10135 (from Alter do Chão; group 2c) is an example for low values for both vegetative and reproductive development;
- accession 10137 (from Portalegre; group 2e) presents low values for vegetative development and late flowering, but high levels of reproductive development;
- accession 10062 (from Arronches; group 2f) is late flowering, has very high levels of vegetative development and small but abundant seeds, leading to high values of seed production;
- among modern cultivars (group 3), 'Myallie' presents higher seed production than others suggesting a better adaptation to local trial conditions.

Molecular diversity

Among the 35 accessions chosen for molecular diversity analyses, only 29 presented results effectively taken for AFLP analysis, while all 35 accessions were analysed by ISSR.

The three ISSR primers considered for analysis originated a total of 25 bands (8.3 bands per primer and 5.6 bands per primer and accession), producing a total of 6 monomorphic bands (24.0% of total). Similarity between accessions (a 35×35 matrix) ranged from a minimum of 0.5185 to a maximum of 0.9767, the average being 0.7681.

Performing a CA on the banding pattern data, a dendrogram was obtained (not shown). In this, two groups of accessions appear clearly separated, with an average dissimilarity of 0.26 between elements of each group. It is possible to relate this grouping pattern with some traits presented by the accessions clustered in those groups. This way, one of these groups clusters exclusively accessions belonging to group 2 of morphological analysis (containing wild accessions). The other group includes also some wild accessions, but it hosts a

sub-group where cultivars and breeding lines gather (groups 1 and 3 of morphological analysis), such as 'Illyarrie', 'Kalya', 'Wonga', 'Jak' and 'Steb'.

The two AFLP primer combinations considered for analysis originated a total of 82 bands (41 bands per primer combination and 32.2 bands per primer combination and accession), with a total of 50 monomorphic bands (61.0% of total). Similarity between accessions ranged from 0.8906 to 0.9925, with as average of 0.9406.

A dendrogram was obtained performing CA. As it was observed with ISSR, AFLP markers also show cultivars and breeding lines as sub-groups, with less diversity, within the diversity found among wild accessions. However, AFLP divides cultivars into two distinct groups. One includes Australian cultivars 'Illyarrie' and 'Myallie' and South African cultivars 'Jak' and 'Steb', while the other clusters European breeding lines (accessions 10160 and 10200) and European-based cultivar 'Frost'.

Bringing AFLP and ISSR data into a single matrix (30 accessions × 107 bands), similarity ranges between 0.8500 and 0.9818, with an average of 0.9071. Performing CA on this matrix, a dendrogram was obtained (Figure 4), where three groups can be pointed out. Two of them (groups A and B) contain all wild accessions. Group A is almost entirely made of accessions geographically collected at Arronches. Group C, clustering apart from the others, includes only cultivars and breeding lines.

Discussion

The morphological analysis of *L. angustifolius* accessions clearly differentiated three groups of accessions, one composed by modern cultivars

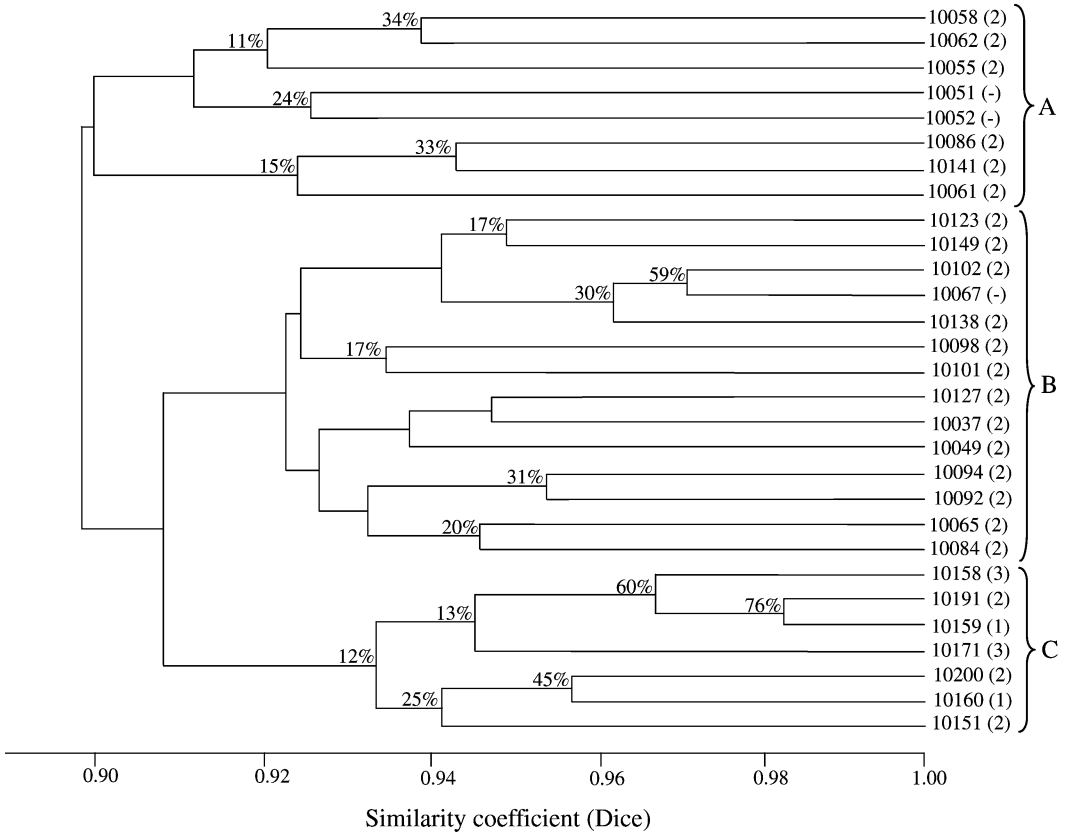

Figure 4. Dendrogram relating accessions used for joint AFLP and ISSR analyses (30 accessions × 107 bands, two AFLP primer combinations and three ISSR primers); calculated upon UPGMA clustering of accessions from a distance matrix; cophenetic correlation coefficient $r = 0.67907$; groups according to morphological characters are shown in brackets.

(except for 'Frost', showing several traits typical of wild accessions, possibly due to poor adaptation to trial conditions), another by wild accessions and a third one composed by cultivars and breeding lines exhibiting different domestication traits (at least, non-shattering pods, large seeds and permeable seed coat), but with vigorous vegetative development, typical of wild accessions.

It is possible to point out several distinctive traits among accessions in any of the domesticated groups. Nevertheless, the group clustering wild accessions revealed the highest levels of diversity. Several sub-groups were differentiated among wild accessions, which could be related in a certain extent to the geographic origin of accessions. This way, accessions coming from higher latitudes tend to present low vegetative and reproductive development indexes and later flowering. Such cline of variation is particularly evident for seed size, with river Tagus being a clear border separating smaller seeded accessions on the North from larger seeded accessions in the South.

Especially for *L. angustifolius* wild germplasm, the accessions pointed out in the results represent the poles of diversity found, orientated according to the axes defined by the main character groups. Certain degrees of independence existing among these axes are materialised in some accessions presenting combinations of traits divergent from the most common patterns. As an example, accession 10062 (from Arronches) exhibits high vegetative development but small seeds. On the other hand, accession 10137 (from Portalegre) is late flowering and has low levels of vegetative development but relatively large seeds.

It should be underlined the contrast found in *L. angustifolius* in relation to the set of characters that are known to combine in *L. albus* geographic cline of variation. Thus, in Iberian *L. albus*, low levels of vegetative development are associated with early flowering and large seeds (Simpson and Martins 1984; Martins 1991). In *L. angustifolius*, however, lower levels of vegetative development come in association with late flowering and small seeds. This crucial difference between *L. albus* and *L. angustifolius* could arise from the rather minor human interference in the evolution of wild *L. angustifolius*. The selection of plants with higher vegetative development in *L. albus* in the Centre and North of Portugal would be both a consequence of the higher water availability and the need to use plants for forage as well as for seed production. *Lupinus angustifolius* from the Centre and North, on the opposite, evolved not in farming lands but especially in marginal uncultivated although disturbed areas, with a more mountainous distribution, as compared to *L. albus*. This way, *L. angustifolius*, not profiting neither the higher water availability as for *L. albus* nor the human selection pressure, would have evolved (in the Centre and North) to less vegetatively developed forms. Late flowering (as compared to South) is a direct consequence of lower average temperatures in the Centre and North.

Three wild accessions arise from the previously described cline of variation. These are accessions 10144 (from Alegrete), 10133 (from Viana do Alentejo) and 10122 (from Castelo Rodrigo). They present thousand seeds weight values over 120 g, matching the description of the subspecies *angustifolius* as proposed by Franco (1971). Large seeds are often found in some wild lupin species (such as *L. atlanticus* Gladst., *L. cosentinii* Guss., *L. digitatus* Forsk., *L. palaestinus* Boiss. and *L. pilosus* Murr.) and in some wild Iberian and Moroccan *L. angustifolius* accessions (Gladstones and Crosbie 1979). This was suggested to be the consequence of very early pre-agricultural repetitive use of these plants by men, thriving in disturbed areas surrounding human settlements (Gladstones 1998). This repetitive use would consequently have originated the selection of large seeded forms, later abandoned, as new, more input-factor responsive crops were developed.

The results of molecular analyses (ISSR and AFLP) show a high base of genetic diversity among wild *L. angustifolius* accessions. These techniques showed cultivars and breeding lines as sub-groups clustering within the wider genetic diversity of wild accessions. This is an evidence for the narrow basis of genetic diversity on which modern cultivars and breeding lines are built, which was not so evident when analysing morphological diversity. Such discrepancy between molecular and morphological analyses leads to the hypothesis that an important proportion of *L. angustifolius* genomes may be composed by non-coding regions, although important morphological differences can be due to very minute molecular changes. Thus, part of the genetic diversity revealed by molecular markers would be silent diversity (or at least, a non-evident diversity).

Comparing the molecular techniques, the levels of divergence found using ISSR were larger than those obtained with AFLP, which again suggests that a higher proportion of ISSR markers are placed in non-coding regions of the genomes.

These results alert for the need to preserve wild germplasm, where larger levels of genetic diversity can be found. The recent domestication of this crop and its cultivation far from the natural distribution area, together with the levels of morphological and molecular diversity found in this study, are encouraging factors leading to the prediction that further use of wild germplasm for breeding purposes can largely benefit the crop.

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